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(54) Title: METHOD FOR PREPARING TEMPLATE DNA FROM CELL CULTURES

(57) Abstract

The present invention provides a simple, rapid and reliable method for sequencing nucleic acid inserts directly from a plasmid DNA which comprises the steps of: spinning down an overnight cell culture to obtain a pellet; resuspending the pellet; heating the resuspended pellet; and separating the insoluble cell debris from the DNA-containing supernatant, whereby the DNA-containing fraction is useful as template DNA for automated sequencing.

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METHOD FOR PREPARING TEMPLATE DNA FROM CELL CULTURES

Field of the Invention

The present invention relates generally to the field of automated sequencing procedures, and more specifically to a method for sequencing plasmid DNA inserts directly from cell lysates.

Background of the Invention

Automated (or manual) sequencing of DNA is a key tool in scientific research, particularly in the search for as yet unidentified genes, particularly human genes. The techniques for automated sequencing reactions are now conventional, and generally employ the apparatus and processes described by manufacturers such as Applied Biosystems Inc. of Foster City, California. However, the demand for improved DNA sequencing methodologies is spurred in large part by the Human Genome Initiative, which is a worldwide research effort that has the goal of analyzing the structure, organization and sequence of human DNA to locate and identify all human genes. As a result, there is a great demand for high-volume sequencing of DNA.

The quality of automated (or manual) DNA sequencing is dependent on the quality of template DNA used in the sequencing reactions. A number of different procedures are currently used to make template DNA from bacterial cells. Such procedures include the miniprep procedure involving lysozyme, an alkaline lysis procedure, boiling, and the Qiagen® column procedure.

As one example, the miniprep procedure [H. C. Birnboim and J. Doly, Nucl. Acids. Res., 7(6):1513-1523 (Aug. 1979)], with lysozyme is performed by treating plasmid-containing cells with lysozyme to weaken the cell wall, and then lysing the cells completely with sodium dodecyl sulfate (SDS) and NaOH. By choosing the ratio of cell suspension to NaOH solution carefully, a reproducible alkaline pH value is obtained, which may be controlled by including glucose as a pH buffer. Chromosomal DNA is then selectively denatured and when the lysate is neutralized by acidic sodium acetate, the chromosomal DNA renatures and aggregates, and can be removed by centrifugation. Plasmid DNA is then recovered from the supernatant by ethanol precipitation.

Alternatively, P. R. Musich and W. Chu, BioTechniques, 14(6):958-960 (1993) describe a hot alkaline lysis procedure for plasmid DNA isolation, which involves modification of the above-described Birnboim and Doly method, the primary advantage of which appears to be removal of contaminating RNA from the sample.

See, also, the procedure of D. Ish-Horowicz and J. F. Burke, Nucl. Acids. Res., 9(13): 2989-2998 (1981), which combines the Birnboim and Doly method with the method of Davis et al, Meth. Enz., 68:404-411 (1979).

Another DNA template preparation process is the rapid boiling method for preparing bacterial plasmids, in which the bacteria are pelleted and resuspended in buffer and lysozyme. The solution is then boiled for 15-40 seconds and the resulting insoluble clot of genomic DNA and debris is removed by centrifugation. Plasmids are then recovered from the supernatant by isopropanol precipitation [see, e.g., D. S. Holmes and M. Quigley, Anal. Biochem., 114:193-197 (1981)]. A further modification of this procedure has been recently reported [see, M. S. Rajeevon and C. L. Bassett, Biotechnique, 16(3) (1994)]. In this protocol, Triton X-100 and lysozyme are added to the overnight culture directly and the mixture is boiled for 1 minute. The cells were removed by spinning and the DNA was isolated from the supernatant by isopropanol precipitation.

Still another template preparation procedure is the QIAGEN® process which involves the following steps. After harvesting and resuspension, bacterial cells are lysed in NaOH/SDS, in the presence of RNAse A. The lysate is neutralized by the addition of acidic potassium acetate, which causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate. The precipitated debris is removed by centrifugation, leaving cleared lysate. This cleared lysate must then be purified, desalted and concentrated, before it is ready for sequencing.

Any of these above described procedures produces DNA of a quality desirable for final sequencing of identified genes or DNA sequences of interest. However, these processes are quite costly as well as time-consuming, and not readily automatable, due to the expense of the required reagents and the labor-intensive nature of the procedures.

There thus exists a need in the art for methods for preparing DNA templates which are inexpensive and suitable for rapid sequencing of large numbers of DNA samples.

Summary of the Invention

In one aspect, the present invention provides a method for rapidly preparing DNA for automated sequencing (i.e. template DNA) which is isolated from a cell culture. This procedure involves growing cell cultures in a tube or multi-well plate and centrifuging or spinning the cultures down with a conventional micro-centrifuge. By spinning the cell culture, a cell-containing pellet and supernatant (containing culture media and other contaminants) are obtained. The supernatant is discarded and the pellet is (optionally) washed with a buffer. The pellet is then resuspended in water and heated for sufficient time to lyse the cells typically 1 to 10 minutes. The resulting insoluble cell debris is separated from the fraction which contains DNA. Preferably, this separation step is performed via centrifugation. The resulting DNA fraction is then useful as the template for automated sequencing.

In another aspect, the invention provides a method for sequencing nucleic acid inserts (e.g., plasmid DNA) directly from cell lysates in an automatic cycle sequencer. This method involves preparing the DNA template by the method of the invention, preferably mixing the template-DNA containing fraction with the automatic cycle sequencing premix and primers, and sequencing the DNA template.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Detailed Description of the Invention

The present invention provides a quick, reliable, readily automatable and inexpensive procedure for preparing template DNA for automated cycle sequencing procedures. This method avoids the use of enzymes, chemicals and other organic solvents common in other known procedures for making template DNA.

The method of the invention provides a quick, simple hot water lysis procedure for making DNA templates for automated DNA sequencing. The resulting DNA templates are of a quality sufficient for the sequencing of large numbers of clones. More specifically, the method of the invention yields template DNA of sufficient quality to obtain at least 300 base pairs of useful sequence data.

The method of the invention is particularly well adapted for use in preparing DNA templates for use with automatic sequencers, including those commercially available from Applied Biosystems, Inc. (ABI), both by primer and terminator chemistries, or from Pharmacia, among other commercial vendors. In order to initially screen hundreds or thousands of clones by automated DNA sequencing techniques, it is not necessary to obtain 100% correct sequencing data for each and every sample.

An error rate of 5-6% can be tolerated in such initial sequencing runs. In comparison to the DNA template preparation processes of the prior art which produce DNA templates of a quality sufficient for final screening, the process of the present invention avoids expending the excess time and resources which are unnecessary for initial screening.

The invention may desirably be applied to screen clones or cell cultures, preferably those which contain high copy plasmid DNA. As used herein, a "plasmid" is an extrachromosomal DNA element capable of replication independent of the host cell chromosome, particularly in bacteria. The term "high copy plasmid" means any plasmid which contains more than one copy of a desired gene or which is itself present in the cell in quantities of two or more.

Additionally, the method of the invention is useful in screening cultures of recombinant host cells which have been transfected or transformed with a selected nucleic acid sequence. As used herein, a "recombinant host cell culture" is any cell culture which has been engineered to contain a recombinant vector or other transfer DNA. As used herein a "vector" is a DNA molecule employed to transfer a foreign gene into a cell.

Suitable vectors and host cells are well known in the art and can be readily obtained from commercial and academic sources. Examples of suitable vectors include pUC 18/19, pBluescript IIKS+ (Stratagene, La Jolla, CA), pcDNA I (Invitrogen, San Diego, CA), the pGEM series, (Promega, Madison, WI), and PCR 2000 (Stratagene, La Jolla, CA). Examples of suitable host cells include bacterial cell cultures, including *Escherichia coli*, particularly strains XL1 Blue, C600, HB101, DH1, DH5alpha, MM294C1+, and JM109 cells (all available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD), INVαF' cells (Stratagene), and SOLR cells, which are commercially available (Stratagene; Invitrogen, San Diego, CA; or BRL, Bethesda, MD). Other suitable host systems, include yeast, mammalian, *Streptomyces* and insect cells, including Baculovirus and Drosophila systems.

The particular vector and host system used is not a limiting factor for this invention. Such systems and their uses, are well-known in the art. See, generally, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1990). Further, it is anticipated that this method will be useful in connection with bacteriophage plaques.

The method of the invention is particularly useful for screening the large numbers of cDNA clones which are generated in human genesequencing efforts, to obtain an "Expressed Sequence Tag" or EST or other sequencing tags derived from

genomic DNA. By "EST" is meant a sequence of about 150 to about 300 sequential nucleotides of a longer sequence obtained from a cDNA library prepared from a selected cell, cell type, tissue or tissue type, which longer sequence corresponds to an mRNA of a gene found in that library. An EST is generally DNA. A single DNA library made from a single tissue type is presently anticipated to provide at least about 3000 different ESTs. Further background and information on the construction of ESTs is described in M. D. Adams et al, Science, 252:1651-1656 (1991) and International Application Publication Number WO/93/00353.

The starting materials for the method of the invention are not limited to any particular cell culture, stage of cell growth, or type of growth media. Preferably the starting materials are isolated from bacteria cell culture. The cell culture preferably has a density of at least about 0.8 O.D. unit at 600 nm. It is presently preferable for transformed bacterial cells to be grown in either terrific Broth or Luria Broth (LB) cultures. Preferably, the culture size is at least about 40 µl, particularly when the cells are grown overnight in the LB broth. If bacteriophage is the desired source of template materials, it would be introduced into appropriate host cells, e.g., XL-1 Blue cells, and these cells would be used to make the lysates, as described herein. Thus, the method of the invention may be applied to a selected cell culture, grown using conventional techniques, as discussed above.

In the practice of the method of the invention, a bacterial cell culture having a cell density as defined above is concentrated. Most preferably, this concentration step involves spinning or centrifuging the culture to obtain a pellet. For example, about 40 to about 50 microliter (µL) of a 1-5 mL *E. coli* culture grown for between about 12 to about 16 hours (e.g. an overnight culture) and containing high copy plasmid is spun at between about 3000 to about 15,000 revolutions per minute (rpm) for between about 1 to about 10 minutes. Presently preferred conditions are spinning at about 13,000 rpm for 1 minute. Alternatively, the cells can be spun down at relatively low speeds (2000-5000 rpm), for a longer time period (15-30 minutes). Advantageously, this method may be performed in a 1.5 mL Eppendorf tube or in a 9G - well plate. However, these parameters may be readily adjusted by one of skill in the art for scale-up or as needed or desired. The supernatant which contains media contaminants and cell debris is discarded. Alternatively, although less efficient, the cells may be concentrated by other means, e.g., filtration.

The cell pellet is then washed to remove the traces of media. This washing step preferably involves resuspending the cells in an appropriate amount, e.g. about 200 µl,

of saline, preferably 1 x PBS. The resuspended cells are then spun down and the supernatant which contains the excess media is discarded.

The cell pellet is then resuspended, preferably in an appropriate amount of sterile, distilled water (e.g. about 10 µl), and optionally, vortex mixed. If desired, the pellet may alternatively be resuspended in other appropriate solutions, e.g. 5 X TAC5 buffer [Applied Biosystems, Inc.], PCR buffer, or any other solution which does not interfere with PCR sequencing reactions.

After resuspension of the cell pellet, the mixture is heated to about 95°C to about 100°C for between about 1 to about 10 minutes. Presently preferred conditions for this step are about 100°C for about 5 minutes. This heating step may be performed in a boiling water bath, heating block with water, or by other suitable means. Advantageously, the heating lyses the cells and the DNA is released in aqueous solution. If desired, one of skill in the art could readily adjust the conditions for the heating step, e.g., by taking into account the cell type, among other variables.

Following heating, the cellular debris resulting from lysis is separated from the lysate, which is the fraction of the mixture which contains the template DNA. Currently, the preferred separation step involves subjecting the mixture to a second spinning step. This spinning step is preferably performed for between about 1 to about 10 minutes, at the same speeds as set forth above for the first spinning step. The desirable conditions for this step are spinning at about 13,000 rpm for about 3 minutes. The supernatant which contains the template DNA can then be removed and used directly in an automated sequencer according to conventional methods described by the manufacturer.

Alternatively, the separation step may be performed by filtration. In such a situation, the cell debris would be collected in the filter and the filtrate could be used in an automatic sequencer.

Desirably, prior to use in an automatic sequencer, the fraction of the mixture containing the DNA template, (e.g. a suitable sample size of the supernatant or the filtrate) is mixed with cycle sequencing premix and primers, according to manufacturer's instructions. For example, according to ABI's instructions, 10 µL of the lysate supernatant is added to 10 µL of cycle sequencing premix containing dNTPs, sequencing primer, Taq polymerase enzyme and reaction buffer.

The automatic sequencing reactions are then performed in accordance with conventional techniques, e.g., according to Applied Biosystems' standard procedures using either DyeTerminator or the DyePrimer chemistries. In one example, for DyePrimer chemistry procedures, 10 µL of the bacterial lysate (lysate supernatant) was

divided into four equal volumes (2.5 µL each). After completing the cycle sequencing reactions, samples are purified and treated according to Applied Biosystems' (or other automatic sequencer manufacturer's) standard sequencing procedures. In another example, about 10 µl of the lysate is combined with the standard terminator mix and 1 µL each of either the universal forward primer (F) or the universal reverse primer (R) and this mixture is subjected to the standard thermal cycling conditions according to ABI's instructions. Optionally, an agent may be added to improve PCR or cycle sequencing reaction efficiency. For example, DMSO, when added at 5% final concentration to the cycle sequencing mix, has been found to improve sequencing results when added to the cycle sequencing reaction. Alternatively, glycerol, when added at 10% final concentration to the cycle sequencing mix, has also been found to improve sequencing results.

Example 1 below illustrates that template DNA prepared using the method of the invention compares favorably to template DNA obtained by three other methods, which are much more time consuming and expensive to prepare. The quality of template DNA prepared from alkaline lysis, boiling, and QIAGEN® prep procedures was purer than the template DNA prepared by the hot water lysis method of the present based upon electrophoresis and ethidium bromide staining (on a 1% agarose gel). The template DNA prepared by the method of the invention appeared to contain significant amounts of RNA. However, when the template DNA prepared by each of these procedures was subjected to DyeTerminator cycle sequencing with a universal forward primer or universal reverse primer, the sequencing data for the DNA prepared by the method of the invention (i.e., hot water lysis) was substantially equivalent to other DNA samples.

The method of the present invention is particularly desirable for the sequencing of multiple samples simultaneously. This aspect of the invention involves growing the cultures in 96-well plates using conventional techniques and spinning the cultures down with the well plate spinning rotor head. Suitable well plates and well depths can be readily determined by one of skill in the art. For example, the Beckman 96 well plate having 1 mL deep wells is particularly well suited for application of the method of the invention. The supernatants can be discarded using, e.g., a multi-tip pipetman or a robotic system such as Beckman Biomek, and all of the samples can be processed by the procedure described above.

The following examples illustrate the performance of the method of the invention and the comparison of the present invention with other known methods for DNA template preparation. Briefly, the method of the invention makes use of the

following general protocol. From an overnight (e.g., 12-18 hour) bacterial (e.g., *E. coli*) culture containing a high copy plasmid, 40-50 µl are removed to a 1.5 mL Eppendorf tube. The cells are spun out in a microcentrifuge at 13,000 rpm for 1 minute, and the supernatant is removed and discarded. To wash the cells, the cell pellet is resuspended in 200 µl of 1X PBS buffer, spun down for 1 minute at 13,000 rpm and the supernatant is removed and discarded. (The washing step is optional, not essential.) The cell pellet is then resuspended in 12 µl of sterile distilled water, and heated at 100°C for 5 minutes to lyse the cells. The lysed cells are spun again at 13,000 RPM for 3 minutes, and the supernatant, i.e., the water lysate, containing the DNA is removed to a fresh tube.

9.5 µl of the lysate is combined with the standard terminator mix (Applied Biosystem, Foster City, CA) and 1 µl of forward or reverse primer and this mixture is subjected to the standard thermal cycling conditions according to ABI's instructions. For the primer chemistry which is carried out in four separate tubes, the lysate is divided into four samples, as would be done for a DNA template prepared according to prior art methods. After cycling, the samples are purified and treated as according to the automatic sequencer manufacturer's directions.

The examples which follow are illustrative only and are not to be construed as limiting the present invention.

Example 1 - Preparation of Template DNA

In order to compare the method of invention to other known methods, the pGEM vector DNA (taken out of Applied Biosystem's sequencing kit) was transformed into the INValpha F' cells (Invitrogen, San Diego, CA) using standard techniques. These cells were inoculated into the LB (Luria Broth, 40 mL) media and the culture was grown overnight (16 hours) at 37°C. The culture was then divided into four equal portions and the template DNA was prepared by four different methods.

In the first method, the DNA was made using the method of invention (hot water lysis) essentially as described above. However, the starting volume was about 200 µl. Starting with a larger volume of cell culture gave more template DNA for use in multiple sequencing reactions. Specifically, in this case 200 µl of the overnight culture (in 1.5 ml Eppendorf tube) was spun down at 13,000 rpm for 1 minute using the table top micro-centrifuge and the supernatant was removed by aspiration and discarded. In order to wash the cells, the cell pellet was resuspended in 200 µl of 1X PBS buffer and spun down again at 13,000 rpm for 1 minute. The supernatant was

once again removed by aspiration and discarded. The cell pellet was now resuspended in 50 µl of the sterile distilled water and the suspension was heated at 100°C for 5 minutes (to lyse the cells) using the heating block. The suspension was spun down at 13,000 rpm for 10 minutes and the supernatant removed carefully and saved in a fresh tube. About 10 µl of this lysate was used in DyeTerminator cycle sequencing procedure using Applied Biosystem's protocol.

Additional samples of template DNA were prepared from these cultures using the alkaline lysis method essentially as described in Birnboim and Doly, cited above; the boiling preparative method essentially as described in Holmes and Quigley, cited above, and the Qiagen preparative method essentially as described in the manufacturer's protocol.

While template DNA was obtained using the method of the invention in about 20 minutes, the alkaline lysis method required about 2½ hours, the boiling preparative method required about 2½ hours, and the Qiagen method required about 3 hours to produce the template DNA.

DNA samples prepared by each method were sequenced using Applied Biosystem's DyeTerminator cycle sequencing procedure with a universal forward primer (refer to ABI manual for DyeTerminator chemistry). In all the sequencing reactions, 5% DMSO was added. The use of DMSO has been reported to improve the DNA sequencing reactions, perhaps due to decreased inter- and intra-strand reannealing [P. R. Winship et al, Nucleic Acids Research, 17:1266 (1989)]. Sequencing data was analyzed by the Applied Biosystem's 373 A analysis software (Fluorescent signals are transformed into signal peaks representing each base, which in turn are used to generate the sequence data (refer to the ABI sequencing Manual)). Comparison of the sequencing data showed that the sequence data obtained for 50 to 350 bases from DNA prepared by hot water lysis procedure (method of the invention) was as good as the other DNA samples prepared by other methods.

Example 2 - Sequencing from PCR 2000 vector DNA/INValpha F' cells

In this example, the TGF-beta receptor type II gene obtained from human liver cDNA by PCR technology was subcloned in the PCR 2000 vector ®(Hoffman LaRoche) and the DNA was transformed in the INValpha F' cells (Invitrogen, San Diego, CA). In order to obtain the sequence information of the gene insert, transformed INValpha F' cells were grown overnight (18 hours) in 10 ml of the LB media at 37°C. DNA was made using the method of the invention essentially as described above in general protocol. Specifically, 45 µl of the overnight culture was

spun down at 13,000 rpm for 3 minutes and the supernatant was discarded. The cell pellet was washed with 100 µl of the 1X PBS buffer by resuspending the cells, spinning at 13,000 rpm for 1 minute and discarding the supernatant. The cell pellet was then resuspended in 10 µl of the distilled water and heated at 100°C for 5 minutes. The suspension was spun down at 13,000 rpm for 10 minutes and 9.5 µl of the supernatant was carefully removed. This water lysate (containing template DNA) was then used for automated sequencing. According to this method, DNA was ready for sequencing in 20 minutes. The DNA was also made using the Qiagen procedure using manufacturers' protocol (this procedure took about 3 hours). DNA samples from both procedures were sequenced using DyeTerminator chemistry as per ABI's protocol. Analysis of the sequence data was done using the ABI software. Sequence data from both of the DNA preparations matche the published DNA sequence of the TGF beta receptor gene. However, the method of the present invention saves time, requires fewer reagents, and provides comparable results.

Example 3 - Sequencing from pUC 18 DNA/MM294C1+ Cells

The gene insert in the pUC 18 vector (BRL, Bethesda, MD) was also sequenced by transforming plasmid DNA in MM294C1+ cells using the method of the invention as described above under general protocol. Specifically, transformed MM294C1+ cells were inoculated in 10 mL of the LB media and were grown at 37°C for 16 hours. 100 µl of the overnight cell culture was spun down at 13,000 rpm for 3 minutes and the supernatant was discarded. The cells were washed with 200 µl of 1X PBS buffer as described in Example 1 and then they were resuspended in 20 µl of sterile distilled water. The suspension was heated at 100°C for 5 minutes in order to lyse the cells. After spinning the suspension at 13,000 rpm for 10 minutes, supernatant was removed carefully and 9.5 µl of this water lysate was then used in automated sequencing reactions. Sequence as analyzed by the ABI software was in complete agreement with the published data.

Example 4 - Sequencing from PCR Script Vector/XL-1 Blue Cells

A synthetic gene was cloned in the PCR Script vector and the ligation mixture was transformed in the XL-1 Blue cells (Stratagene, La Jolla, CA). Plasmid DNA was made using the method of the invention as follows. Transformed XL-1 Blue cells were inoculated in 50 mL of LB media and were grown overnight (18 hours) at 37°C. For making the template DNA, 200 µl cell culture was spun down at 13,000 rpm for 3 minutes using the table top micro-centrifuge. Supernatant was discarded and the cell

pellet was washed with 200 µl of 1X PBS buffer. After spinning, the supernatant was discarded and the cell pellet was resuspended in 50 µl of distilled water. The suspension was heated at 100°C for 5 minutes to lyse the cells and then spun down at 13,000 rpm for 10 minutes. The supernatant was collected and 9.5 µl of this supernatant was used in Applied Biosystems' DyeTerminator sequencing procedure. Just for comparison, plasmid DNA was made by the published alkaline lysis method. Sequencing of both of these DNA samples and the resulting data analysis using ABI software gave the expected sequence data (i.e., exact nucleotide sequences for the synthetic gene).

Example 5 - Sequencing of Multiple Samples: pUC 19 DNA/TB-1 Cells

The plasmid DNA was made by the method of the invention essentially as described above in the detailed description. Specifically, about 40 bacterial clones (TB-1 cells had previously been infected with pUC19 DNA using conventional techniques; the cells are available from the ATCC) were individually inoculated in 5 mL of LB media in separate tubes and the cell cultures were grown overnight (16 hours) at 37°C. For preparing plasmid DNA, 50 µl of the overnight culture from each tube was spun down at 13,000 rpm for 3 minutes. The supernatant was removed by aspiration and was discarded. In this experiment, the cell pellet was not washed with 1X PBS buffer as in previous experiments. After removing the LB media, the cell pellet from each tube was resuspended in 10 µl of distilled water, heated at 100°C for 5 minutes in a boiling water bath and spun down at 13,000 rpm for 10 minutes. The supernatants were carefully removed from each tube and then used in automated DyeTerminator sequencing. Sequencing the lysates and the data analysis was performed using Applied Biosystems's protocol. In each case, at least 300 base pairs of sequence information were read and about 98% of the DNA samples prepared by the method of invention gave good sequencing data. A good sequence is defined as between 95-98% identical to the expected nucleotide sequence data. The remaining 2-5% error represents the inability of the software to correctly identify a DNA base, which is probably a function of DNA quality. (For comparison, high quality DNA should give >99% accurate sequence).

This experiment indicates that one can delete "the washing step with 1X PBS" from the method of the invention and still obtain good sequencing data for DNA made by the method of the invention.

Example 6 - Sequencing of Multiple Samples: pBluescript Vector DNA/XL-1 Blue Cells

This is yet another example where a large number of clones were sequenced. In this experiment 27 different bacterial clones were used to make the template DNA by the method of the invention. For each sample, transformed XL-1 Blue cells were inoculated in 10 mL of the LB media in separate tubes and the cultures were grown at 37°C overnight (18 hours). To prepare the template DNA, 200 µl of the overnight culture from each tube was spun down at 13,000 rpm for 3 minutes and the supernatants were discarded. The cell pellet in each tube was washed with 200 µl of 1X PBS buffer as described in Example 1. The cell pellet in each tube was then resuspended in 50 µl of sterile distilled water and heated at 100°C for 5 minutes using the heating block. The cell suspensions were spun down at 13,000 rpm for 10 minutes and the supernatants were collected. 9.5 µl of the supernatant from each tube was used in automated sequencing. Data was analyzed using the ABI software and gave 300 base pairs of sequence for each sample.

Example 7 -Sequencing from pSelect Vector DNA/MM294C1+ Cells

The pSelect Vector is mainly used for doing the site directed mutagenesis in which a gene sequence can be altered at a very specific location using a procedure supplied by the manufacturer (Promega, Madison, WI). Two mutations in a synthetic gene (Antibody cassette) were corrected using this procedure. In order to verify the mutations, it was necessary to sequence this gene. For making template DNA, transformed MM294C1+ cells were inoculated in 10 mL of the LB media and the culture was grown overnight (16 hours) at 37°C. 200 µl of the overnight culture (in a 1.5 mL Eppendorf tube) was spun down at 13,000 rpm for 3 minutes. The supernatant was removed by aspiration and was discarded. The cell pellet was washed with 1X PBS buffer and then resuspended in 50 µl of distilled water. The suspension was heated at 100°C for 5 minutes and then spun down at 13,000 rpm for 10 minutes. The supernatant was carefully collected and kept in a fresh tube. 9.5 µl of the supernatant was used in the automated sequencing reaction using the universal forward primer. Sequencing data was analyzed by ABI 373A software and showed correct the mutational changes.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of

skill in the art. Such modifications and alterations to the processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A method for preparing template DNA for automated or direct cycle sequencing which comprises:

(a) centrifugation of a cell culture containing plasmid DNA to obtain a pellet;

(b) resuspending the pellet;

(c) heating the resuspended pellet for sufficient time to lyse the cells from the pellet; and

(d) separating cell debris from a DNA-containing fraction.

2. The method according to claim 1 wherein the spinning step (a) is performed at about 3000 revolutions per minute (rpm) to about 15,000 rpm for between about 1 minute to about 10 minutes.

3. The method according to claim 1 wherein the spinning step is performed at about 13,000 rpm for about 1 minute.

4. The method according to claim 1 wherein the pellet of step (a) is resuspended in water.

5. The method according to claim 1 wherein the heating step (c) is performed at about 95°C to about 100°C for between about 1 minute to about 10 minutes.

6. The method according to claim 5 wherein the heating step (c) is performed at about 100°C for about 5 minutes.

7. The method according to claim 1 wherein said separation step (d) is performed via centrifugation or filtration.

8. The method according to claim 1 wherein said separation step (d) comprises the steps of spinning the heated pellet of step (c) for about 5 minutes to about 10 minutes at about 3000 revolutions per minute (rpm) to about 15,000 rpm and removing the supernatant, which supernatant contains template DNA.

9. The method according to claim 1 wherein said separation step (d) comprises the steps of filtering the heated pellet of step (c) and collecting the filtrate, which filtrate contains the template DNA.

10. A method for preparing template DNA for direct cycle sequencing which comprises:

(a) centrifugation at a rate of about 3000 to 15000 rpm for between about 1 minute to about 10 minutes of a cell culture containing plasmid DNA to obtain a pellet;

(b) resuspending the pellet in water;

(c) heating the resuspended pellet at a temperature of between about 95°C to about 100°C for between about 1 minute to 10 minutes to lyse the cells in the pellet; and

(d) spinning the heated pellet of step (c) for about 5 to about 10 minutes at about 3000 revolutions per minute (rpm) to about 15,000 rpm and removing the supernatant, which supernatant contains template DNA.

11. A method for preparing template DNA for direct cycle sequencing which comprises:

(a) centrifugation at a rate of about 3000 to 15000 rpm for between about 1 minute to about 10 minutes of a cell culture containing plasmid DNA to obtain a pellet;

(b) resuspending the pellet in water;

(c) heating the resuspended pellet at a temperature of between about 95°C to about 100°C for between about 1 to 10 minutes to lyse the cells in the pellet; and

(d) filtering the heated pellet of step (c) and collecting the filtrate, which filtrate contains template DNA.

12. Template DNA prepared by the method according to claim 1.

13. Template DNA prepared by the method according to claim 10.

14. Template DNA prepared by the method according to claim 11.

15. A method for sequencing plasmid DNA isolated from a cell for use in an automated sequencer comprising the steps of:

(a) centrifugation of a cell culture to obtain a pellet;

(b) resuspending the pellet;

(c) heating the resuspended pellet for sufficient time to lyse the cells from the pellet;

(d) separating cell debris from a template DNA-containing fraction; and

(e) sequencing the template DNA in said fraction.

16. The method according to claim 15 wherein said separating step (d) comprises the steps of centrifuging the heated pellet of step (c) and removing the supernatant which contains the template DNA.

17. The method according to claim 15 wherein prior to sequencing step (e), the supernatant is mixed with cycle sequencing premix and primers.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04539

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12N 1/08, 1/20, 15/00; C07H 17/00
US CL : 435/6, 252.3, 270, 320.1; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 252.3, 270, 320.1; 536/23.1; 935/1, 16, 19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Innis et al., "PCR PROTOCOLS A GUIDE TO METHODS AND APPLICATIONS", published 1990 by Academic Press, Inc. (N.Y.), pages 14-15; see page 15.	1, 12-14 --- 2-11, 15-17
X Y	Maniatis et al., "Molecular Cloning A Laboratory Manual", published 1982 by Cold Spring Harbor Laboratory (N.Y.), pages 86-96; see pages 89-91.	1, 12-14 --- 3-11, 15-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, U. S. A., Volume 86, issued December 1989, Sorge et al., "ExoMeth sequencing of DNA: Eliminating the need for subcloning and oligonucleotide primers", pages 9208-9212; see pages 9208, 9209, 9211.	1-11, 15-17
X		12-14